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Increased Hepatic Nicotinamide N-Methyltransferase Activity as a Marker of Cancer Cachexia in Mice Bearing Colon 26 Adenocarcinoma

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When a cachexigenic subclone (clone 20) of murine colon 26 adenocarcinoma was transplanted into female BALB/c mice, hepatic NNMT activity continued to increase until death in proportion to progressive carcass weight loss, a marker of cancer cachexia. On the other hand, noncachexigenic subclone (clone 5)-transplanted mice showed neither increase of NNMT activity nor carcass weight loss. Among cytostatic fluorinated pyrimidines, 5′-dFUrd could inhibit the increase of NNMT activity and prevent weight loss in mice bearing clone 20. On the other hand, 2′-dFUrd did not show these effects. 5-FUra and Tegafur inhibited the increase of NNMT activity at higher concentrations. These findings suggest that the levels of hepatic NNMT activity are closely associated with the degree of weight loss, and they appear to be a useful marker of cancer cachexia.

Key words: Nicotinamide N-methyltransferase — Cancer cachexia — Liver — Colon 26 adenocarcinoma

Ado-Met:NNMT (EC 2.1.1.1) catalyzes the N-methylation of nicotinamide and other pyridines.[1)] Ado-Met functions as a methyl donor for this reaction. NNMT is predominantly localized in the mammalian liver.[2–4)] Recently, the NNMT gene was cloned and characterized.[5)]

Cancer cachexia is a complex syndrome characterized by body weight loss, anorexia, depletion of muscle and fat tissue, anemia and some altered blood metabolic parameters (e.g., hypoglycemia), etc.[6)] Cancer cachexia is responsible for both decreased response to therapy and shortened survival.[7)]

Our previous study showed that hepatic NNMT activity increased after inoculation of various tumors into mice.[8)] However, the mechanisms by which NNMT activity increases are not yet understood.

Colon 26 adenocarcinoma is a chemically induced, murine colon-adenocarcinoma cell line.[9)] This tumor has been shown to induce marked cachexia (estimated in terms of carcass weight loss) in mice when the tumor mass is still relatively small, a situation similar to that found clinically.[10, 11)] There are two subclones of colon 26 adenocarcinoma; one is clone 20 with a remarkable cachexia-inducing potency, and the other is clone 5 without such potency.[12)]

In order to determine the relationship between the level of hepatic NNMT activity and the degree of cancer cachexia, we examined these parameters in a murine model using the aforementioned two clones. In the mice bearing the original colon 26 adenocarcinoma, cachexia was prevented by 5′-dFUrd in spite of the ineffectiveness of many other cytostatics.[13)] Thus, we examined whether levels of NNMT activity were correlated with the prevention of cachexia by 5′-dFUrd in this model. The present study showed that there was a direct correlation between levels of hepatic NNMT activity and the degree of cancer cachexia.

MATERIALS AND METHODS

Chemicals [^{3}H-methyl]Ado-Met (80 Ci/mmol) was purchased from Amersham International plc, Buckinghamshire, UK. Nicotinamide and 1-methylnicotinamide chloride were from Sigma, St. Louis, MO. 5′-dFUrd was donated by Nippon Roche K. K., Tokyo. 2′-dFUrd and 5-FUra were obtained from Wako Chemical, Tokyo. Tegafur was donated by Taiho Pharm. Co., Ltd., Tokushima, Japan. NNMT cDNA was a kind gift from Dr. R.M. Weinshilboum, Mayo Foundation, Rochester, MN.[4)]

Animals Eight-to-ten-week-old, female BALB/c mice were obtained from Japan Shizuoka Laboratory Animal Center (Hamamatsu). They were given standard laboratory chow (Funabashi F-2, Chiba) and water *ad libitum*.

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[5] Abbreviations used: NNMT, nicotinamide N-methyltransferase; 5′-dFUrd, 5′-deoxy-5-fluorouridine; 2′-dFUrd, 2′-deoxy-5-fluorouridine; 5-FUra, 5-fluorouracil; Ado-Met, S-adenosyl-L-methionine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1, interleukin-1; IL-6, interleukin-6.

The experiments were performed in accordance with the Guide for Animal Experimentation, Inohana Campus, Chiba University.

Tumors Two subclones derived from the murine colon 26 adenocarcinoma cell line, clones 20 and 5, were donated by Nippon Roche Research Center (Kamakura, Kanagawa).[12] The cells of each clone were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), penicillin G (100 units/ml) and streptomycin (100 μg/ml) in a humid atmosphere containing 5% (v/v) CO_2.

Tumor inoculation Single-cell suspensions were made by means of trypsin treatment. Then 1×10^6 cells were inoculated s.c. into the right inguinal flank of each mouse.

Measurement of body wasting (cancer cachexia) Whole body weight, and the length (*a*) and width (*b*) of the tumors were measured every other day. The tumor weight was estimated by using the formula $ab^2/2\times F$, where *F* is the correction factor determined by comparing the actual tumor weight with the calculated tumor volume ($ab^2/2$) in a separate experiment. Carcass weight was calculated by subtracting tumor weight from whole body weight.

Fluorinated pyrimidine administration 5′-dFUrd, 2′-dFUrd, 5-FUra, and Tegafur were each dissolved in 0.5% carboxymethylcellulose. The clone 20-bearing mice were each given a fluorinated pyrimidine p.o. every day from day 8 after tumor inoculation for 6 days.

Assay of NNMT activity The assay was based on the conversion of nicotinamide to radioactive N^1-methylnicotinamide by [^{3}H-methyl]Ado-Met, which serves as a methyl donor. Mice were killed by cervical dislocation followed by decapitation. Livers excised from mice were homogenized with a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) in 10 m*M* Tris-HCl buffer (pH 7.8) containing 10 m*M* 2-mercaptoethanol and 1m*M* phenylmethylsulfonyl fluoride and then centrifuged at 105,000*g* for 1 h. The supernatant obtained was assayed for NNMT activity as described by Nakagawa *et al.*[14] After incubation at 37°C for 5 min, the supernatant from a reaction mixture was spotted onto Whatman 3MM filter paper, and the paper was developed. Radioactivity in the 1-methylnicotinamide spot was measured using an Aloka LSC-703 liquid scintillation spectrometer. The heat-denatured liver homogenate was used as a blank. Protein content of the liver extract was measured by the method of Lowry *et al.*[15] using bovine serum albumin as a standard.

Northern hybridization Total RNAs were extracted from livers by the guanidine thiocyanate method.[16] Ten micrograms of total RNAs was electrophoresed on 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N; Amersham), and hybridized with NNMT cDNA as a probe using a multiple megaprime labeling kit (Amersham). The levels of GAPDH mRNA expression were used to normalize the amount of RNA loaded. Quantitation of the autoradiographic intensities was performed by laser densitometry using a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, Frisco).

Statistical analysis Mean and SE of all parameters determined in this study were calculated. Statistical analysis was performed by using Student's *t* test, with $P<0.05$ as the criterion of significance.

RESULTS

Correlation between levels of hepatic NNMT activity and carcass weight loss in mice bearing subclones of colon 26 adenocarcinoma In order to examine the correlation between the levels of hepatic NNMT activity and the degree of cachexia, clone 20 (cachexigenic) and clone 5 (non-cachexigenic), subclones of murine colon 26 adenocarcinoma, were inoculated into mice. *In vivo*, clone 20 and clone 5 grew at similar rates until at least day 14, after the tumor masses became visible at day 6 (Fig. 1a). In agreement with a previous study,[17] clone 20-bearing mice showed carcass weight loss from day 8 (Fig. 1b), while clone 5-bearing mice did not until at least day 14. Hepatic NNMT activity of the mice bearing clone 20 began to increase at day 8 and continued to increase progressively until day 14, even if the mice became moribund before that time point (Fig. 1c). On the other hand, levels of hepatic NNMT activity of the mice bearing clone 5 did not show any change through day 14. The results indicate that there was a good correlation between the increase of hepatic NNMT activity and carcass weight loss in the mice bearing clone 20.

Expression of hepatic NNMT mRNA in mice bearing clone 20 or 5 To investigate whether the increase of hepatic NNMT activity was due to the increase of transcriptional activity, we examined the hepatic NNMT mRNA in mice bearing each clone. Hepatic NNMT mRNA levels in the mice with clone 5 were not different from those of normal mice (Fig. 2, a and b), but the levels in the clone 20 mice were higher than in normal mice. These results indicate that hepatic NNMT activity is regulated by the transcriptional level.

Effects of cytostatic fluorinated pyrimidines on both carcass weight loss and increase of hepatic NNMT activity in clone 20 mice Previous studies showed that 5′-dFUrd could reduce tumor weight and prevent carcass weight loss when it was given to mice bearing the original colon 26 adenocarcinoma. 2′-dFUrd could reduce only the tumor weight, and 5-FUra and Tegafur neither reduced the tumor weight nor prevented the carcass weight loss.[13] Therefore, in order to confirm further the correlation between the increase of hepatic NNMT activity and the degree of cachexia, such fluorinated pyrimidines were

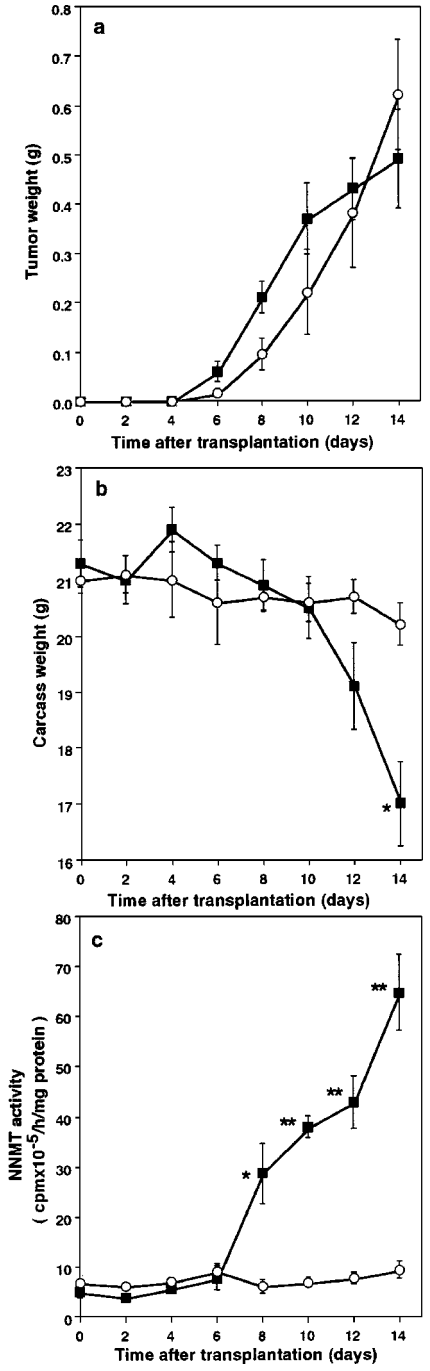

Fig. 1. Tumor weight, carcass weight and hepatic NNMT activity after inoculation (1×10^6 cells) of either clone 20 (■) or clone 5 (○) into mice. (a) Tumor weight and (b) carcass weight were measured in each mouse. At day 14, (c) hepatic NNMT activity was measured in triplicate. Values are mean±SE (bar). Each group consists of five mice. (b) ∗ Significantly different versus day 0 at $P<0.005$. (c) ∗, ∗∗ Significantly different versus day 0 at $P<0.01$ or 0.001, respectively. A significant inverse correlation between carcass weight loss and the increase in NNMT activity was observed ($r=-0.19$, $P<0.001$).

given to mice bearing clone 20. In our study, 5′-dFUrd prevented carcass weight loss at all doses used, although not to a statistically significant degree, and also significantly reduced tumor weight at higher doses ($P<0.05$) (Fig. 3, a and b). On the other hand, 2′-dFUrd neither prevented carcass weight loss nor reduced tumor weight (Fig. 4, a and b). Similarly, 5-FUra also failed to prevent carcass weight loss or reduce tumor weight (Fig. 5, a and b), but Tegafur reduced tumor weight only at a dose of 1.5 mmol/kg (Fig. 6, a and b).

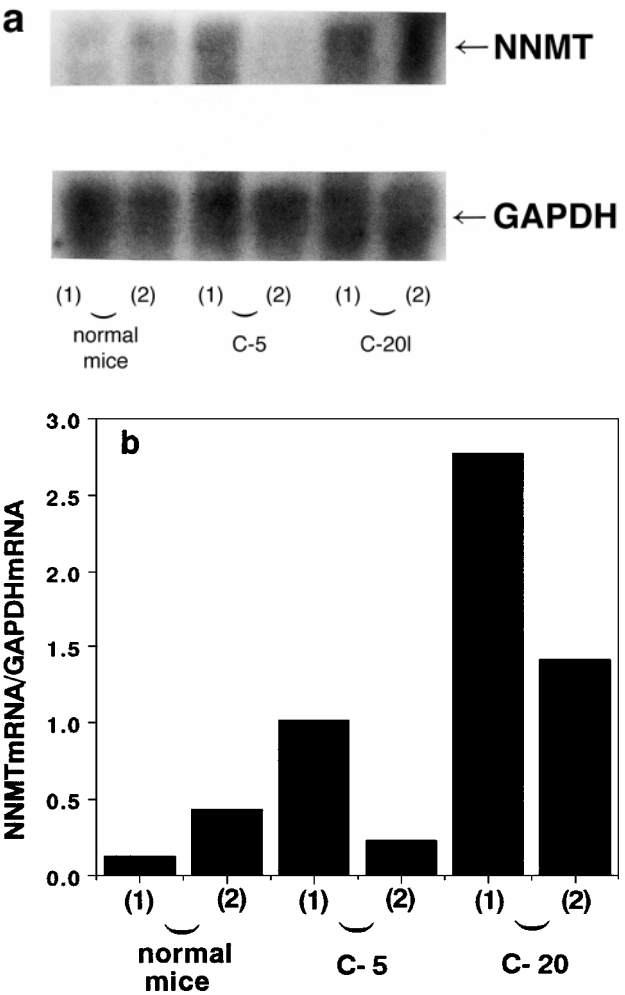

Fig. 2. (a) Expression of NNMT mRNA in the livers of mice at day 14 after inoculation of either clone 20 or clone 5. Northern blot analysis was performed on total RNAs extracted from either normal mice, clone 5-bearing mice or clone 20-bearing mice as described in "Materials and Methods." Representative results from two independent experiments are shown here. (b) Quantitation of densitometric intensities for NNMT mRNA. Data are expressed as the ratios of density for NNMT mRNA relative to GAPDH mRNA.

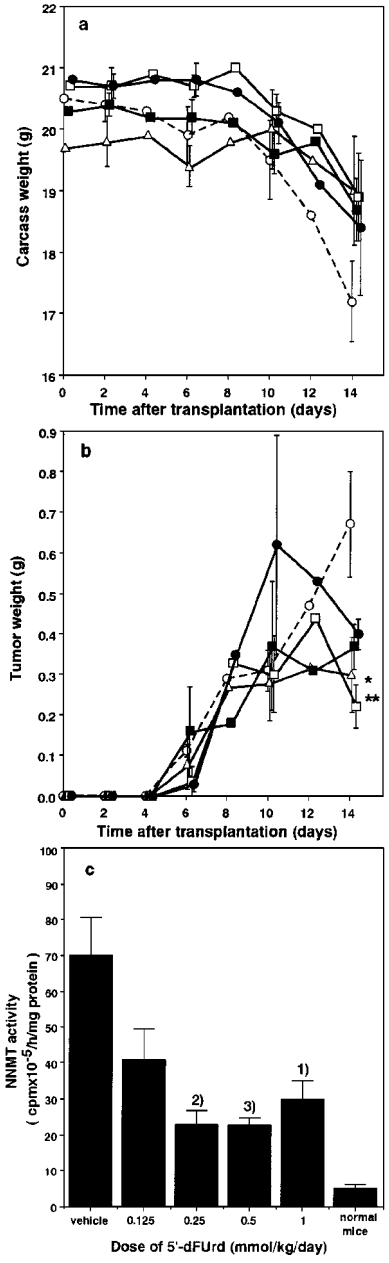

Fig. 3. Effects of 5′-dFUrd on carcass weight, tumor weight and hepatic NNMT activity. Five mice were implanted with clone 20. Changes of (a) carcass weight and (b) tumor weight were monitored, and the mean±SE (bar) was calculated. 5′-dFUrd (○ vehicle, ● 0.125, □ 0.25, ■ 0.5, △ 1 mmol/kg/day) was administered p.o. to mice every day from day 8 for 6 days. (c) Hepatic NNMT activity was measured in triplicate at day 14, and values are mean±SE (bar). One mouse given 5′-dFUrd (0.125 mmol/kg/day) died on day 14. (b) *, ** Significantly different versus vehicle group at $P<0.05$ or 0.02, respectively. (c) 1), 2), 3) Significantly different versus vehicle group at $P<0.02$, 0.01 or 0.005, respectively. A significant inverse correlation was observed between the recovery of carcass weight loss and levels of NNMT activity in mice after administration of 5′-dFUrd ($r=-0.96$, $P<0.005$).

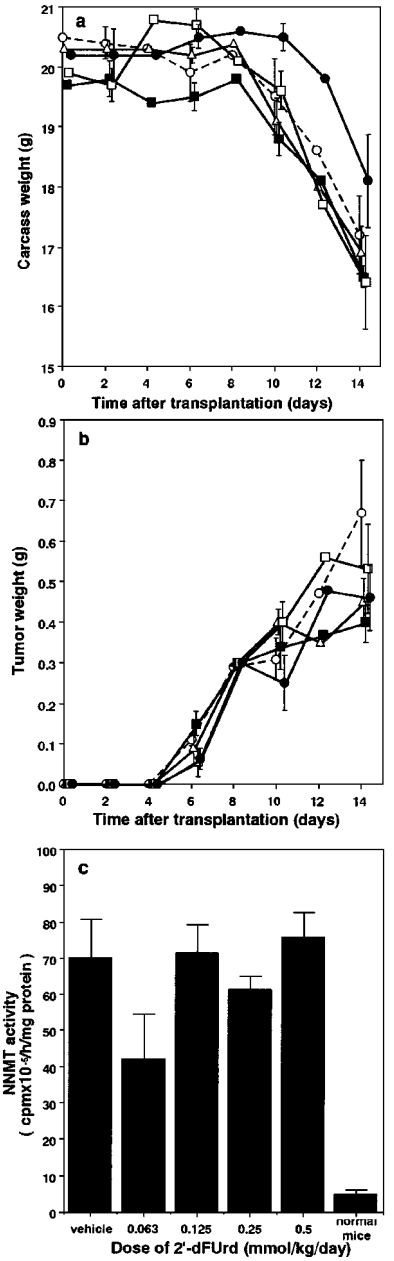

Fig. 4. Effects of 2′-dFUrd on carcass weight, tumor weight and liver NNMT activity. Five mice were implanted with clone 20. Changes of (a) carcass weight and (b) tumor weight were monitored, and the mean±SE (bar) was calculated. 2′-dFUrd (○ vehicle, ● 0.063, □ 0.125, ■ 0.25, △ 0.5 mmol/kg/day) was administered p.o. to mice every day from day 8 for 6 days. (c) Hepatic NNMT activity was measured in triplicate at day 14, and values are mean±SE (bar). No significant correlation was observed between the recovery of carcass weight loss and levels of NNMT activity in mice after administration of 2′-dFUrd.

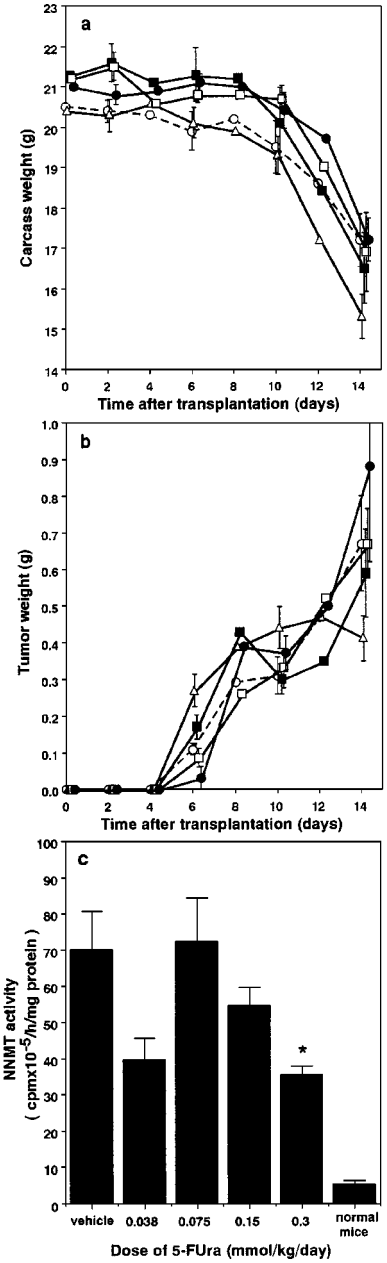

Fig. 5. Effects of 5-FUra on carcass weight, tumor weight and liver NNMT activity. Five mice were implanted with clone 20. Changes of (a) carcass weight and (b) tumor weight were monitored, and the mean±SE (bar) was calculated. 5-FUra (○ vehicle, ● 0.038, □ 0.075, ■ 0.15, △ 0.3 mmol/kg/day)was administered p.o. to mice every day from day 8 for 6 days. (c) Hepatic NNMT activity was measured in triplicate at day 14, and values are mean±SE (bar). One mouse administered 5-FUra (0.15 mmol/kg/day) died on day 14. ∗ Significantly different versus vehicle group at $P<0.05$. No significant correlation was observed between the recovery of carcass weight loss and levels of NNMT activity in mice after administration of 5-FUra.

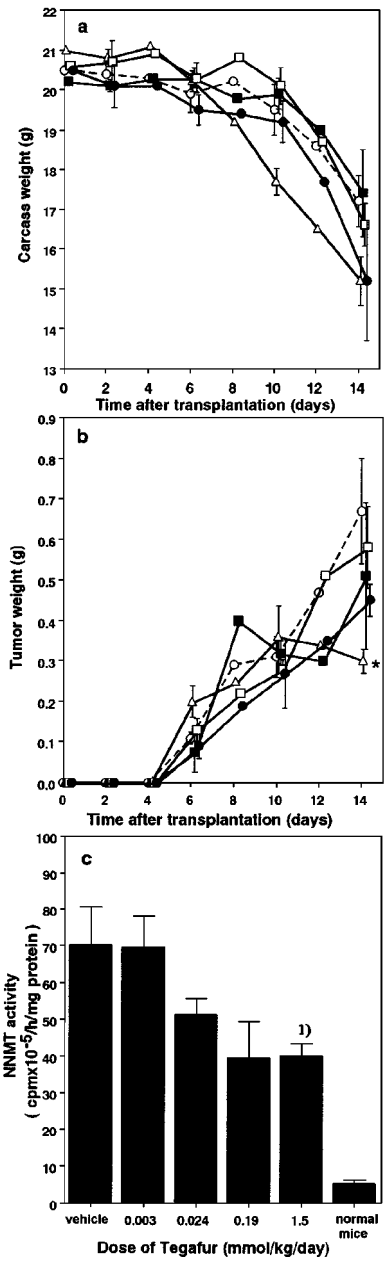

Fig. 6. Effects of Tegafur on carcass weight, tumor weight and hepatic NNMT activity. Five mice were implanted with clone 20. Changes of (a) carcass weight and (b) tumor weight were monitored, and the mean±SE (bar) was calculated. Tegafur (○ vehicle, ● 0.003, □ 0.024, ■ 0.19, △ 1.5 mmol/kg/day) was administered p.o. to mice every day from day 8 for 6 days. (c) Hepatic NNMT activity was measured in triplicate at day 14, and values are mean±SE (bar). One mouse given Tegafur (0.19 mmol/kg/day) died on day 14, and one mouse (0.003 mmol/kg/day) died on day 12. (b) ∗ Significantly different versus vehicle group at $P<0.02$. (c) 1) Significantly different versus vehicle group at $P<0.05$. No significant correlation was observed between the recovery of carcass weight loss and levels of NNMT activity in mice after administration of Tegafur.

Further, we examined the effects of these fluorinated pyrimidines on the increase of hepatic NNMT activity at day 14 in the mice bearing clone 20. We found that 5′-dFUrd inhibited the increase of NNMT activity at all doses except 0.125 mmol/kg (Fig. 3c). However, when 5′-dFUrd was given to normal mice, the levels of NNMT activity did not change (data not shown). 2′-dFUrd did not cause any inhibition (Fig. 4c), and 5-FUra and Tegafur showed inhibition only at doses of 0.3 and 1.5 mmol/kg, respectively (Figs. 5c and 6c). The results of administration of 5′-dFUrd confirmed that the recovery of carcass weight loss is closely associated with inhibition of the increase of hepatic NNMT activity in clone 20-bearing mice.

DISCUSSION

Cancer cachexia is characterized principally by emaciation[18] occurring even at an early stage of the development of malignancy.[19] Body weight loss is observed in about half of untreated cancer patients.[20] Cancer cachexia brings about a deterioration of the quality of life, a decreased response to chemotherapy, and shortened survival.[7, 21] Therefore, it is necessary to understand the mechanisms of the development of cancer cachexia, which are still poorly understood.

Body weight loss can occur even in patients without anorexia.[22] Anorexia was not observed in the clone 20-bearing mice in the present study (data not shown), in agreement with a previous study.[23] Numerous studies aimed at the identification of mediators which induce cachexia have been carried out. Such mediators include toxohormone, a cancer cachectic factor, tumor necrosis factor-α, IL-1 and -6, etc.,[11, 24–33] but none of these mediators has the ability actually to reproduce cachexia.[23, 34–36]

We have observed the enhancement of nucleic acid metabolism in the liver of tumor bearers.[37–41] Our previous study showed that hepatic NNMT activity continued to increase until death in mice bearing Ehrlich ascites tumor,[8] coupled with a decrease in hepatic catalase activity, a putative cachexia marker.[42] The present study also showed that hepatic NNMT activity continued to increase until day 14 in the mice bearing the cachexigenic clone 20 (50% survival: about 18 days[34]). Although tumor growth only started at day 6, weight loss as well as increase in NNMT activity was observed as early as at day 8. On the other hand, neither NNMT activity nor carcass weight changed in the mice with clone 5, which is non-cachexigenic (50% survival: about 65 days[34]). These results demonstrated that the levels of NNMT activity are well correlated with the degree of cancer cachexia (weight loss), but not with tumor growth.

It has been reported that 5′-dFUrd, a prodrug of 5-FUra,[43, 44] exerts potent anticachectic activity, even at doses which allow the growth of colon 26 adenocarcinoma.[13] As a mechanism for the anticachectic effects, it has been proposed that 5′-dFUrd selectively damages tumor cells, in which pyrimidine nucleoside phosphorylase activity[45, 46] as well as IL-6 production is enhanced[47]; 5′-dFUrd is activated to kill tumor cells by pyrimidine nucleoside phosphorylase,[43, 44] while IL-6 is a major cachectic effector.[11] We observed that the weight loss was prevented by 5′-dFUrd administration, although the effect was not statistically significant (Fig. 3a), and only when 5′-dFUrd was administered at higher doses did it reduce tumor weight, but then significantly (Fig. 3b). 5′-dFUrd significantly inhibited the increase of hepatic NNMT activity at a dose of 0.5 mmol/kg, but could not reduce tumor growth at this dose (Fig. 3, b and c). On the other hand, other fluorinated pyrimidines, and particularly 2′-dFUrd, which had no role in the prevention of weight loss, did not inhibit the increase of NNMT activity (Figs. 4, 5 and 6). These results might indicate that the degree of inhibition of the increase of NNMT activity is in proportion to that of prevention of weight loss. On the basis of these findings, we think that the hepatic NNMT activity might be useful as a marker of cancer cachexia.

Moreover, although the inhibition of the increase of hepatic NNMT activity was statistically significant, the prevention of weight loss was not significant in 5′-dFUrd-administered clone 20-bearing mice (Fig. 3, a and c). Therefore, the levels of NNMT activity may reflect the degree of cancer cachexia with greater sensitivity than weight loss. For this reason, it is likely that 5-FUra and Tegafur inhibited the increase of NNMT activity without the prevention of weight loss at doses of 0.3 and 1.5 mmol/kg, respectively (Figs. 5a, 5c, 6a and 6c).

The levels of IL-6 in the sera and tumor tissues were higher in clone 5 mice than in normal mice, but similar to those in clone 20 mice.[12] Recent studies showed that cancer cachexia is caused by IL-6 and other mediator(s) in mice bearing clone 20.[23] In addition, it has been reported that anti-IL-6 antibody partially inhibits weight loss in clone 20 mice.[16] Nonetheless, the effect of anti-IL-6 antibody on hepatic NNMT activity with respect to its relationship with tumor weight and carcass weight remains to be elucidated. An understanding of the NNMT-regulating mechanisms may help to unravel those of the development of cancer cachexia.

Certain clinical features such as anorexia, anemia, etc. are often observed in cancer patients, but useful clinical and laboratory parameters of cancer cachexia have yet to be found. So, it is desirable to establish criteria for the diagnosis of cancer cachexia. A decrease of catalase activity has been revealed in tumor-bearing animals.[48] However, this could be due not only to the tumor burden, but also to the decline of the tumor host. On the other hand, NNMT activity was inversely correlated with the degree

of the cachexic effect, and thus, NNMT activity should be more useful than catalase to measure the degree of the cachexic effect. We propose hepatic NNMT activity as a candidate criterion of cancer cachexia.

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